Deletion of the *Trypanosoma brucei* Superoxide Dismutase Gene *sodb1* Increases Sensitivity to Nifurtimox and Benznidazole[▽]

S. Radhika Prathalingham, Shane R. Wilkinson, David Horn, and John M. Kelly*

*Department of Infectious and Tropical Diseases, London School of Hygiene and
Tropical Medicine, London WC1E 7HT, United Kingdom*

It has been more than 25 years since it was first reported that nifurtimox and benznidazole promote superoxide production in trypanosomes. However, there has been no direct evidence of an association between the drug-induced free radicals and trypanocidal activity. Here, we identify a superoxide dismutase required to protect *Trypanosoma brucei* from drug-generated superoxide.

Parasites of the *Trypanosoma brucei* complex are the causative agents of African trypanosomiasis. Vaccine development against this disease is unlikely, and chemotherapy is unsatisfactory (1, 4): drugs require administration under medical supervision, can fail to eradicate parasitemia, and are often toxic. Melarsoprol, which is used against late-stage disease, kills 5 to 10% of patients (12). Without treatment, trypanosomiasis is fatal. A new approach, currently under trial, is the use of nifurtimox in combination with eflornithine (18, 21). The nitroheterocyclic drugs nifurtimox and benznidazole are normally used to treat *Trypanosoma cruzi* infections. Although effective against acute-stage Chagas' disease, they are of limited use against the chronic stage, which occurs in ~30% of patients, often many years after the initial infection (2, 9). Nifurtimox can be orally administered, is readily absorbed, and crosses the blood-brain barrier. However, side effects include nausea/vomiting and central nervous system toxicity, and use has been associated with genotoxic and carcinogenic effects (10, 19). In addition, some *T. cruzi* strains are refractory to treatment (16). If nifurtimox is to be used against African trypanosomiasis, it is important that both the mechanism(s) of action and the potential for resistance are more fully explored.

One hypothesis for the trypanocidal effects of nifurtimox proposes similarities to the antibacterial activity of other nitrofurans, with drug activation by type I nitroreductases leading to the generation of reactive moieties that promote DNA damage (14, 20). The only trypanosome enzyme reported to mediate this type of two-electron reduction is prostaglandin F2α synthase (13) and then only under anaerobic conditions. A second hypothesis invokes the generation of oxidative stress, following one-electron reduction of the drug by type II nitroreductases. Under aerobic conditions, this promotes redox cycling, with the formation of superoxide anions and drug regeneration (6, 7, 15, 23). However, there is no evidence that these free radicals contribute to

trypanocidal activity. Normally, superoxide anions are detoxified by superoxide dismutases (SODs), a family of antioxidant metalloenzymes. Trypanosome SODs belong to the Fe class, a group restricted to protozoans, prokaryotes, and chloroplasts. *T. brucei* contains four isoforms (8, 11, 25): TbSODA and TbSODC, which are mitochondrial, and TbSODB1 and TbSODB2, which are localized predominantly in the glycosome, a kinetoplastid-specific organelle (3, 17, 24). TbSODB1 and TbSODB2 also display some cytosolic localization, particularly the former (8, 25). Neither TbSODA nor TbSODC is essential for parasite viability, although when TbSODA was down-regulated using RNA interference (RNAi), the bloodstream form of *T. brucei* became sensitive to the superoxide generator paraquat but not to nifurtimox or benznidazole (25). When RNAi was targeted at Tb*sodb1* or Tb*sodb2*, both transcripts were down-regulated, due to their high levels of sequence identity. The effect was lethal, with growth cessation occurring within 24 h.

To assess the contributions of Tb*sodb1* and Tb*sodb2* to oxidative defense and to determine whether they protect against drug-induced free radicals, we investigated the possibility of generating null mutant cell lines. The flanking regions of Tb*sodb1* and Tb*sodb2* are not highly conserved and are sufficiently divergent to facilitate targeted gene knockout. Vectors for this purpose were constructed (Fig. 1) and used to transfect *T. brucei* bloodstream forms, using electroporation (25). Tb*sodb1* was replaced by the genes for puromycin acetyltransferase (*pac*) and blasticidin deaminase (*bla*). Similarly, Tb*sodb2* was replaced by *bla* and the gene that confers phleomycin resistance (*ble*). Targeted deletion was confirmed by Southern analysis (Fig. 1). The Tb*sodb1*- and Tb*sodb2*-null mutants were both viable in culture and did not exhibit significantly altered phenotypes in terms of growth rate or infectivity in BALB/c mice (data not shown).

We further examined the null mutants to determine if they displayed increased sensitivity to nifurtimox, benznidazole, and paraquat. Parasites were seeded at 1×10^4 cells per ml in microtiter plates with 200 μl of growth media containing different drug concentrations and were incubated at 37°C for 48 h (25). Twenty microliters of Alamar Blue was

* Corresponding author. Mailing address: Department of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London WC1E 7HT, United Kingdom. Phone: 44 207 927 2330. Fax: 44 207 636 8739. E-mail: john.kelly@lshtm.ac.uk.
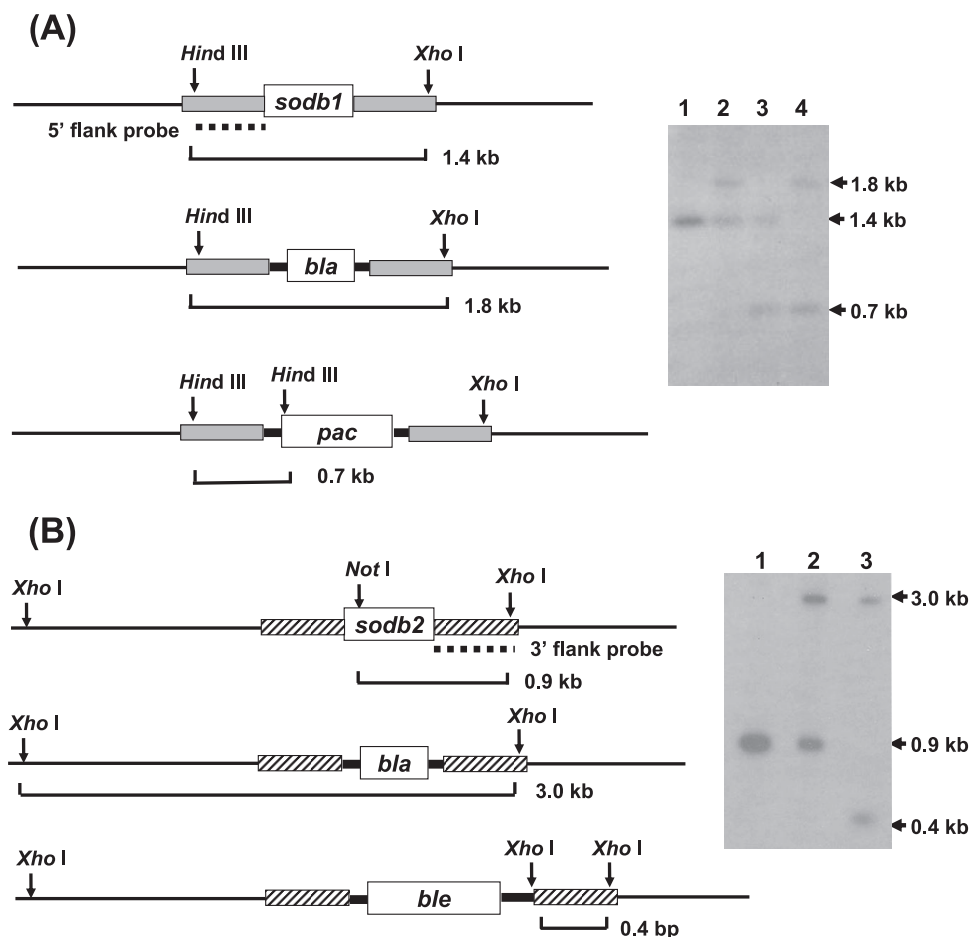
FIG. 1. Targeted replacement of Tb*sodb1* and Tb*sodb2*. (A) Map of the Tb*sodb1* genetic locus, showing the 5′- and 3′-flanking regions (gray boxes) that were used for targeted integration. These were amplified by PCR and cloned to either side of *bla* and *pac* expression cassettes. The bloodstream form of *T. brucei* (Lister 427 strain) was transfected with these constructs (25), and null mutant clones were selected. Genomic DNA was isolated, digested with HindIII/XhoI, and analyzed by Southern hybridization, using a probe specific to the 5′ flank of Tb*sodb1* (indicated by the dashed line). Lane 1, parental cell line; lane 2, *bla* single knockout; lane 3, *pac* single knockout; lane 4, *pac/bla* double knockout. Sizes of the predicted hybridizing fragments are shown below the corresponding genetic maps. (B) Deletion vectors for Tb*sodb2* were constructed as outlined above, using the 5′- and 3′-flanking regions for targeting (striped boxes) and *bla* and *ble* expression cassettes for selection. Following the generation of null mutant clones, genomic DNA was isolated, digested with NotI/XhoI, Southern blotted, and hybridized with a specific 3′-flank probe (dashed line). Lane 1, parental cell line; lane 2, *bla* single-knockout clone; lane 3; *bla ble* double-knockout clone.

then added to each well, and the plates were analyzed by a fluorescent plate reader (Fig. 2). There were no significant differences between the drug concentrations required to inhibit growth of the Tb*sodb2*-null mutant and the parental cell line by 50% (IC_{50}). However, the Tb*sodb1* mutant displayed twofold-, threefold-, and fourfold-increased susceptibility to benznidazole, nifurtimox, and paraquat, respectively (Fig. 2). To confirm that loss of Tb*sodb1* was responsible for the increased drug sensitivity, we reintroduced a copy of the 0.6-kb Tb*sodb1* gene into the null mutants, using pTubEx (5). This vector facilitates integration into the tubulin gene array and contains the neomycin phosphotransferase gene (*neo*) as a selectable marker. Transformants were verified by Southern hybridization, and expression of Tb*sodb1* was confirmed by Northern blotting (Fig. 3). In the complemented cell line, processing of the Tb*sodb1* RNA is mediated by tubulin gene-flanking se-

quences and produces a 0.9-kb transcript. When these cells were reexamined, we found that expression of TbSODB1 from this locus completely reversed the drug sensitivity phenotype in the cases of benznidazole and nifurtimox and partially so in the case of paraquat.

Previously, the limitations of RNAi meant we could not distinguish between TbSODB1 and TbSODB2 in terms of functional significance (25). Furthermore, drug sensitivity studies were restricted by rapid-growth inhibition and cell death. By independently deleting Tb*sodb1* and Tb*sodb2*, we have now demonstrated that, under normal growth conditions, there is functional redundancy in this arm of the *T. brucei* oxidative defense. However, TbSODB1, but not TbSODB2, is required to protect the parasite from superoxide radicals generated by the redox cycling of nitroheterocyclic drugs. As in most organisms, trypanosome SODs are highly compartmentalized (8, 25). This reflects the re-
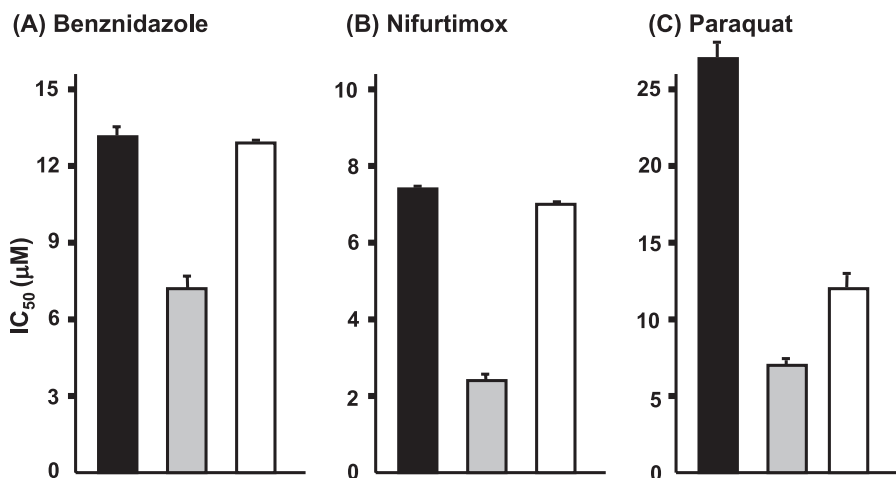
(A) Benznidazole (B) Nifurtimox (C) Paraquat

FIG. 2. Susceptibility of the bloodstream form of *T. brucei* (IC_{50}) to nitroheterocyclic drugs and paraquat. Black bars, parental cell line; gray bars, Tb*sodb1*-null mutants; white bars, complemented cell line. After *T. brucei* cells were grown in media containing different drug concentrations for 48 h under 5% CO_2, the vital stain Alamar Blue (Invitrogen) was added and the plates were incubated for a further 24 h. The IC_{50} values were calculated after final analyses were performed using a fluorescent plate reader (Molecular Devices) at an excitation wavelength of 530 nm, an emission wavelength of 585 nm, and a filter cutoff at 550 nm. The results shown are the means of the results of three experiments ± the standard deviations. The differences between the parental strain and the null mutants were statistically significant in each case ($P < 0.01$), as assessed by Student's *t* test.

stricted ability of superoxide anions to cross biological membranes and the requirement for detoxification to occur at the site of free radical formation. By implication, the subcellular location of an enzyme(s) that mediates the one-electron reduction of nifurtimox and benznidazole, leading to the generation of superoxide radicals, must overlap with TbSODB1. In this context, the observation that TbSODB1 is more abundant in the cytosol than TbSODB2 (8) may be significant. Fe-SODs have been highlighted as potential chemotherapeutic targets because of their parasite specificity (22). The crucial role of TbSODB1 in preventing drug-induced, superoxide-mediated killing suggests that inhibitors targeted at Fe-SODs could act synergistically with nifurtimox.
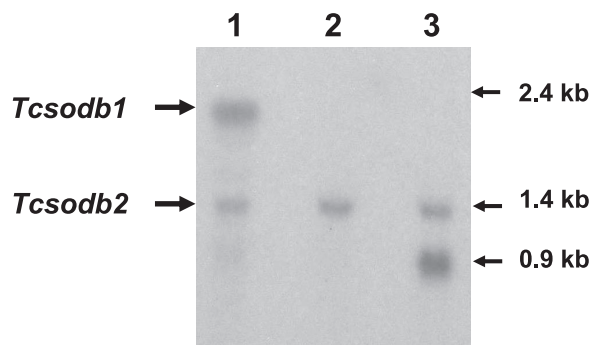

FIG. 3. Autoradiograph of a Northern blot showing complementation of the Tb*sodb1*-null mutant. Lane 1, parental cell line; lane 2, Tb*sodb1*-null mutants; lane 3; complemented cell line. Null mutants were generated as described for Fig. 1. Complementation was achieved by inserting a copy of the Tb*sodb1* gene into the tubulin locus, using the construct pTubEx (5). Ten micrograms of RNA was run on each lane, and hybridization was carried out using a full-length copy of the Tb*sodb1* gene. Transcript sizes are given in kilobases.

REFERENCES

1. **Barrett, M. P., R. J. Burchmore, A. Stich, J. O. Lazzari, A. C. Frasch, J. J. Cazzulo, and S. Krishna.** 2003. The trypanosomiases. Lancet **362:**1469–1480.
2. **Cerecetto, H., and M. Gonzalez.** 2002. Chemotherapy of Chagas' disease: status and new developments. Curr. Top. Med. Chem. **2:**1187–1213.
3. **Colasante, C., M. Ellis, T. Ruppert, and F. Voncken.** 2006. Comparative proteomics of glycosomes from bloodstream form and procyclic culture form *Trypanosoma brucei brucei.* Proteomics **6:**3275–3293.
4. **Croft, S. L., M. P. Barret, and J. A. Urbina.** 2005. Chemotherapy and trypanosomiases and leishmaniasis. Trends Parasitol. **21:**508–512.
5. **Cross, M., R. Kieft, R. Sabatini, A. Dirks-Mulder, I. Chaves, and P. Borst.** 2002. J-binding protein increases the level and retention of the unusual base J in trypanosome DNA. Mol. Microbiol. **46:**37–47.
6. **Docampo, R., and A. O. Stoppani.** 1979. Generation of superoxide anion and hydrogen peroxide induced by nifurtimox in *Trypanosoma cruzi.* Arch. Biochem. Biophys. **197:**317–321.
7. **Docampo, R.** 1990. Sensitivity of parasites to free radical damage by antiparasitic drugs. Chem. Biol. Interact. **73:**1–27.
8. **Dufernez, F., C. Yernaux, D. Gerbod, C. Noel, M. Chauvenet, R. Wintjens, V. P. Edgcomb, M. Capron, F. R. Opperdoes, and E. Viscogliosi.** 2006. The presence of four iron-containing superoxide dismutase isozymes in trypanosomatidae: characterization, subcellular localization, and phylogenetic origin in *Trypanosoma brucei.* Free Radic. Biol. Med. **40:**210–225.
9. **Garcia, S., C. O. Ramos, J. F. V. Senra, F. Vilas-Boas, M. M. Rodrigues, A. C. Campos-de-Carvalho, R. Ribeiro-dos-Santos, and M. B. P. Soares.** 2005. Treatment with benznidazole during the chronic phase of experimental Chagas' disease decreases cardiac alterations. Antimicrob. Agents Chemother. **49:**1521–1528.
10. **Gorla, N. B., O. S. Ledesma, G. P. Barbieri, and I. B. Larripa.** 1989. Thirteenfold increase of chromosomal aberrations non-randomly distributed in chagasic children treated with nifurtimox. Mutat. Res. **224:**263–267.
11. **Kabiri, M., and D. Steverding.** 2001. Identification of a developmentally regulated iron superoxide dismutase of *Trypanosoma brucei.* Biochem. J. **360:**173–177.
12. **Kennedy, P. G.** 2004. Human African trypanosomiasis of the CNS: current issues and challenges. J. Clin. Investig. **113:**496–504.
13. **Kubata, B. K., Z. Kabututu, T. Nozaki, C. J. Munday, S. Fukuzumi, K. Ohkubo, M. Lazarus, T. Maruyama, S. K. Martin, M. Duszenko, and Y. Urade.** 2002. A key role for old yellow enzyme in the metabolism of drugs by *Trypanosoma cruzi.* J. Exp. Med. **196:**1241–1251.
14. **McCalla, D. R., A. Reuvers, and C. Kaiser.** 1971. Breakage of bacterial DNA by nitrofuran derivatives. Cancer Res. **31:**2184–2188.
15. **Moreno, S. N. J., R. P. Mason, and R. Docampo.** 1984. Reduction of nifurtimox and nitrofurantoin to free radical metabolites by rat liver mitochon-

dria: evidence of an outer membrane-located nitroreductase. J. Biol. Chem. **259:**6298–6305.

16. **Murta, S. M. F., R. T. Gazzinelli, Z. Brener, and A. J. Romanha.** 1998. Molecular characterization of susceptible and naturally resistant strains of *Trypanosoma cruzi* to benznidazole and nifurtimox. Mol. Biochem. Parasitol. **93:**203–214.

17. **Parsons, M.** 2004. Glycosomes: parasites and the divergence of peroxisomal purpose. Mol. Microbiol. **53:**717–724.

18. **Priotto, G., C. Fogg, M. Balasegaram, O. Erphas, A. Louga, F. Checchi, S. Ghabri, and P. Piola.** 2006. Three drug combinations for late-stage *Trypanosoma brucei gambiense* sleeping sickness: a randomized clinical trial in Uganda. PLoS Clin. Trials **1:**e39. [Online.]

19. **Rodriques Coura, J., and S. L. de Castro.** 2002. A critical review on Chagas disease chemotherapy. Mem. Inst. Oswaldo Cruz **97:**3–24.

20. **Streeter, A. J., and B. A. Hoener.** 1988. Evidence for the involvement of a nitrenium ion in the covalent binding of nitrofurazone to DNA. Pharmacol. Res. **5:**434–436.

21. **TDR News.** March 2006. Workers on African trypanosomiasis unite. www.who.int/tdr/publications/tdrnews/news76/tryps.htm.

22. **Turrens, J. F., and J. M. McCord.** 2005. The iron-containing superoxide dismutases of Trypanosomatidae. Free Radic. Biol. Med. **40:**193–195.

23. **Viode, C., N. Bettache, N. Cenas, R. L. Krauth-Siegel, G. Chauviere, N. Bakalara, and J. Perie.** 1999. Enzymatic reduction studies of nitroheterocycles. Biochem. Pharmacol. **57:**549–557.

24. **Wilkinson, S. R., R. Prathalingham, M. C. Taylor, D. Horn, and J. M. Kelly.** 2005. Vitamin C biosynthesis in trypanosomes: a role for the glycosome. Proc. Natl. Acad. Sci. USA **102:**11645–11650.

25. **Wilkinson, S. R., R. Prathalingham, M. C. Taylor, A. Ahmed, D. Horn, and J. M. Kelly.** 2006. Functional characterisation of the iron superoxide dismutase gene repertoire in *Trypanosoma brucei.* Free Radic. Biol. Med. **40:**198–209.